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**RF1 knockout allows ribosomal incorporation of unnatural amino acids at multiple sites.**

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**Public Summary:**

We created a novel bacterial strain that has one release factor knocked out. This strain enables non-natural amino acids to be incorporated into proteins with high efficiency, therefore large amounts of mutant proteins containing non-natural amino acids can be produced for scientific research or therapeutic usage. In addition, this strain uniquely allows non-natural amino acids to be incorporated at multiple positions into the same protein, thus opening new opportunities for protein research and the generation of novel protein properties that are not available with natural building blocks.

**Scientific Abstract:**

Stop codons have been exploited for genetic incorporation of unnatural amino acids (Uaas) in live cells, but their low incorporation efficiency, which is possibly due to competition from release factors, limits the power and scope of this technology. Here we show that the reportedly essential release factor 1 (RF1) can be knocked out from *Escherichia coli* by 'fixing' release factor 2 (RF2). The resultant strain JX33 is stable and independent, and it allows UAG to be reassigned from a stop signal to an amino acid when a UAG-decoding tRNA-synthetase pair is introduced. Uaas were efficiently incorporated at multiple UAG sites in the same gene without translational termination in JX33. We also found that amino acid incorporation at endogenous UAG codons is dependent on RF1 and mRNA context, which explains why *E. coli* tolerates apparent global suppression of UAG. JX33 affords a unique autonomous host for synthesizing and evolving new protein functions by enabling Uaa incorporation at multiple sites.

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